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【Inventor】

【Address】c/o MITSUBISHI KAGAKU BIO-CLINICAL LABORATORIES, INC., 3-30-1 Shimura, Itabashi-Ku, Tokyo

【Name】Kyouichi SEKINE

【Inventor】

【Address】c/o MITSUBISHI KAGAKU BIO-CLINICAL LABORATORIES, INC., 3-30-1 Shimura, Itabashi-Ku, Tokyo

【Name】Tatsuo UETAKE

【Applicant】

【Ref. NO.】591122956

【Name】MITSUBISHI KAGAKU BIO-CLINICAL LABORATORIES, INC.

【Agent】

【Ref.No.】100088904

【Patent Attorney】

【Name】Takashi SHOJI

【tel.】03-3864-6572

【Selected agent】

【Ref.No.】100124453

【Patent Attorney】

【Name】Yuriko SUKENOBU

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【List of filed document】

【Document name】Claim 1

【Document name】Specification 1

【Document name】Figure 1

【Document name】Abstract 1

【Document Name】CLAIMS

【Claim 1】A method for inspecting a level of migration of fat-soluble vitamins and/or fat-soluble food factors contained in ingested health supplements, drugs or foods into a body, comprising analyzing fat-soluble vitamins and/or fat-soluble food factors in saliva.

【Claim 2】A method for inspecting effect or action of an administered therapeutic agent on biosynthesis and metabolism of fat-soluble vitamins and/or fat-soluble food factors, comprising analyzing fat-soluble vitamins and/or fat-soluble food factors in saliva as indicators.

【Claim 3】 A method for inspecting of disease condition, comprising analyzing as an marker fat-soluble vitamins and/or fat-soluble food factors in saliva.

【Claim 4】 The method according to any of claims 1 to 3, wherein saliva is parotid saliva.

【Claim 5】 The method according to claim 4, wherein the parotid saliva is collected using a saliva collecting tool which collects parotid saliva selectively and quantitatively.

【Claim 6】 The method according to claim 5, wherein the saliva collecting tool has (a) a collection part comprising an absorber for absorbing saliva irreversibly and (b) a quantification part for quantifying the amount of saliva collected to the absorber.

【Claim 7】 The method according to claim 6, wherein the

saliva collecting tool has further a storage container part for holding a saliva preservative solution in which the absorber with the absorbed saliva is dipped to preserve.

【Claim 8】 The method according to claim 7, wherein the saliva preservative solution is a water-soluble organic solvent.

【Claim 9】 The method according to any of claims 1 to 8, comprising:

(a) a process for extracting the collected saliva, the absorber with the absorbed saliva, and/or the preservative solution with the preserved absorber, with a water-soluble organic solvent,

(b) a process for using the extract as an analysis sample to separate the fat-soluble vitamins and/or the fat-soluble food factors by high-performance liquid chromatography, and

(c) a process for detecting the separated fat-soluble vitamins and/or the fat-soluble food factors.

【Claim 10】The method according to any of claims 1 to 9, wherein the fat-soluble vitamins and/or the fat-soluble food factors are at least one substance selected from a group consisting of CoQ10, lycopene, β-carotene and tocopherol.

【Claim 11】A method for screening drugs or health supplements, wherein the method uses a method according to any of claims 1 to 10.

【Document Name】SPECIFICATION

【TITLE OF INVENTION】METHOD FOR INSPECTING A LEVEL OF MIGRATION OF FAT-SOLUBLE VITAMIN AND/OR FAT-SOLUBLE FOOD FACTOR INTO A BODY BY SALIVA ANALYSIS

【TECHNICAL FIELD】

[0001]

The present invention relates to a method for inspecting a level of migration of fat-soluble vitamins and/or fat-soluble food factors into a body by saliva analysis.

【Background of the Invention】

[0002]

Dietary Supplement, which is translated as "health supplement" or "nutritional supplement", is ingested mainly to supplement nutritional components such as vitamin, mineral, and amino acid which are apt to be scrimpy in everyday life (hereinafter, may be referred to "supplement", "health supplement" or "nutritional supplement"). In the U.S., there are laws about supplements, and supplements are positioned as a new genre different from both foods and pharmaceuticals, and have been approved to label with a Health Claim of the activity to reduce the risk of diseases.

Analysis of in vivo migration of an administered substance to a living body, which is usually executed by determining contents such as blood level and urine level,

needs specimen collection executed by a specific facility such as a medical institution. Therefore it has been inappropriate to analyze in vivo migration of a nutritional component contained in a nutritional supplement or the like.

Saliva may also be a specimen but has inadequate to use as a specimen for a method for analyzing in vivo migration of a nutritional component contained in an ingested health supplement. It is partly because a method for collecting specimen has not been established to give a reproducible value.

CoQ10, which is a nutritional component comprised in the nutritional supplements and constantly presents in mitochondrias, is a coenzyme involved in energy production in which ADP is converted to ATP by oxidation from a reduced form of ubidecarenol ($CoQ10H_2$) to an oxidized form of ubidecarenone (CoQ10) in the electron transport system. In a living body, CoQ10 is not mainly present in the blood, but in the tissues, and about 90% of CoQ10 is present as a reduced form in cell membranes and phospholipid double membranes in organelles. The CoQ10 can be synthesized through rate-limiting enzyme reactions in common with the cholesterol biosynthesis pathway in a body, therefore its synthesis is supposed to be inhibited in a patient treated with a statin base drug although it is not a vitamin. A Statin base drug with higher lipophilicity is supposed to

have higher degree of inhibitory potency against the synthesis, and ingestion of CoQ10 is considered to be useful for avoiding the adverse effects by the drug. Clinical trials by its combination therapy have been already performed since the year before last in the U.S.

Tocopherol (vitamin E), which is a nutritional component comprised in the health supplements, is a major substance to prevent a lipid radical from oxidation, because tocopherol radicals collides with each others to eliminate the radical. A large abundance of tocopherol lowers incidence of collision among the radicals to cause the radical to remain longer. However, a reduced form of CoQ10 in vivo is believed to be potently associated with the tocopherol radicals to eliminate the radical.

Meanwhile, in the developed countries, the mortality rate in myocardial infarction and cerebral infarction potentially resulting from arteriosclerosis is being increased, and the number of diabetics potentially resulting from the lifestyle is rising, and in Japan, diabetes is tops in the novel pathogenesis resulting in hemodialysis patients.

These so-called life-style related diseases are considered to be strongly affected by dietary habit and strongly involve increased oxidative stress in vivo, therefore aggressive ingestion of a substance against oxidative stress is recommended as nutritional supplements.

However, a so-called non-patient, who is not at a stage to visit a hospital, loses a chance to receive health management by a doctor to fall in a worsened symptom before visiting the hospital. This too late treatment may contribute to increased number of patients.

There has been no report about concentrations and contents of CoQ10 in saliva and salivary gland associated with salivation. It may be because saliva levels of a vitamin impossible to synthesize in a human body and a so-called supplement capable of being ingested by diet are easily assumed to be influenced by amount and sort of the foods and drinks left in the oral cavity, the concentrated existing substances derived from various bacteria and fungi, and additionally plaque bacteria flora on the surface of a tooth.

But, fat-soluble vitamins and/or fat-soluble food factors such as Tocopherol,Licopen and CoQ10 etc. about all of total saliva, parotid saliva , submandibular gland and sublingual gland saliva had not been able to analysis.

In addition, although fat-soluble β-carotene which is a provitamin of vitamin A has been known to show a positive correlation between the serum level and the whole saliva level (for example, refer to Nonpatent documents No. 1 and 2), the object of the reporters in Nonpatent documents No. 1 is "to confirm and verify assumption that an ingested β-carotene is useful for maintenance of oral hygiene by

enhancing production and secretion of an antibacterial protein such as glycoprotein or lysozyme in the salivary gland", it was finding on a new physiological activity of β-carotene. The reporters in Nonpatent documents No. 1 decided that their targeted physiological activity could not be detected. They thought it is significantly meaningful to assay β-carotene in total saliva, which was not their basic purpose. Further, there were leaved in a condition be unable to assay β-carotene in parotid saliva. Then, there had been thought it is not a proper method to inspect migration of β-carotene into a body by total saliva analysis because total saliva is contaminated large volume of parotid saliva.

Thus, the present invention is significant to have presented a method of assay β-carotene in parotid saliva that had been thought to be unable way, and to have presented an idea that just parotid saliva is more suitable biological sample for inspecting a level of migration of fat-soluble vitamins and/or fat-soluble food factors into a body. Furthermore, the present invention is particularly significant to have provided a method for analyzing and inspecting a level of migration of fat-soluble vitamins and/or fat-soluble food factors in saliva, especially in parotid saliva.

Nonpatent document No. 1: Int. J. Vitam. Nutr. Res. 1988; 58(2): 171-7 Saliva concentrations of some selected proteins and glycoprotein markers in man after supplementary intake of beta-carotene. Lumikari M. Johansson I., Ericson T., Virtamo J.
Nonpatent document No. 2: Nutr. Cancer 1988; 11(4): 233-41 Effects of excess vitamin A and canthaxanthin On salivary gland tumors. Alam BS, Al am SQ, Weir JC Jr.

【Disclosure of the Invention】

【Problems to be Solved by the Invention】

[0003]

An object of the present invention is to provide a simple means of inspecting a level of in vivo migration of fat-soluble vitamins and/or fat-soluble food factors which are nutritional components contained in health supplements, drugs or foods (hereinafter, may be referred to "health supplement or the like") in ingestion of health supplements or the like.

Means for Solving the Problem

[0004]

The present inventors repeated keen study to solve the above problems, as a result, a selected sample was found to be available as a specimen for inspecting a level of in vivo migration of fat-soluble vitamins and/or fat-soluble

food factors which are nutritional components contained in health supplements or the like, in ingestion of health supplements or the like. Even if the concentration level of analytes in the saliva was approximately 1/10 of the blood level, it was found that assessment was certain. In particular, the present inventors found that β-carotene and other food factors such as lycopene, tocopherol and CoQ10 could be detected and analyzed in parotid saliva supposed to be undetectable as well as whole saliva. Also, the present inventors found that the concentration of CoQ10 or the like in parotid saliva has positive correlation with the blood level but them in whole saliva has not correlation with the blood level, and diligently improved a method of collecting parotid saliva. Unfavorably, an absorber is used for saliva collection to adsorb fat-soluble vitamins and/or fat-soluble food factors in saliva regardless of its material, and fat-soluble vitamins and/or fat-soluble food factors is extracted by using ethanol and is recovered in around 100% level. The present invention is completed based on these findings.

[0005]

Therefore, the present invention comprises:

1. A method for inspecting a level of migration of fat-soluble vitamins and/or fat-soluble food factors contained in ingested health supplements, drugs or foods into a body, comprising analyzing fat-soluble vitamins

and/or fat-soluble food factors in saliva.

2. A method for inspecting effect or action of an administered therapeutic agent on biosynthesis and metabolism of fat-soluble vitamins and/or fat-soluble food factors, comprising analyzing fat-soluble vitamins and/or fat-soluble food factors in saliva as indicators.

3. A method for inspecting of disease condition, comprising analyzing as an marker fat-soluble vitamins and/or fat-soluble food factors in saliva.

4. The method according to any of the above item 1 to 3, wherein saliva is parotid saliva.

5. The method according to the above item 4, wherein the parotid saliva is collected using a saliva collecting tool which collects parotid saliva selectively and quantitatively.

6. The method according to the above item 5, wherein the saliva collecting tool has (a) a collection part comprising an absorber for absorbing saliva irreversibly and (b) a quantification part for quantifying the amount of saliva collected to the absorber.

7. The method according to the above item 6, wherein the saliva collecting tool has further a storage container part for holding a saliva preservative solution in which the absorber with the absorbed saliva is dipped to preserve.

8. The method according to the above item 7, wherein the saliva preservative solution is a water-soluble organic

solvent.

9. The method according to any of the above item 1 to 8, comprising:

(a) a process for extracting the collected saliva, the absorber with the absorbed saliva, and/or the preservative solution with the preserved absorber, with a water-soluble organic solvent,

(b) a process for using the extract as an analysis sample to separate the fat-soluble vitamins and/or the fat-soluble food factors by high-performance liquid chromatography, and

(c) a process for detecting the separated fat-soluble vitamins and/or the fat-soluble food factors.

10. The method according to any of the above item 1 to 9, wherein the fat-soluble vitamins and/or the fat-soluble food factors are at least one substance selected from a group consisting of CoQ10, lycopene, β-carotene and tocopherol.

11. A method for screening drugs or health supplements, wherein the method uses a method according to any of the above item 1 to 10.

Effects of the Invention

[0006]

The methods provided by the present invention allow

analysis of fat-soluble vitamins such as tocopherol and β-carotene and/or fat-soluble food factors such as lycopene and CoQ10 from saliva, and facilitate simple inspection on a level of in vivo migration of the fat-soluble vitamins and/or fat-soluble food factors which are nutritional components contained in health supplements or the like, in ingestion of the health supplements or the like. Namely, the methods in the present invention could confirm that the target fat-soluble vitamins and/or fat-soluble food factors in saliva increased with the intake of the health supplements or drugs. Consequently, saliva allows noninvasive evaluation of nutritional values of foods or the like, and assessment of degree of in vivo migration of fat-soluble vitamins and/or fat-soluble food factors by ingestion of health supplements or drugs, and noninvasive assessment of effect or action of an administered therapeutic agent on biosynthesis or metabolism of the fat-soluble vitamins and/or fat-soluble food factors. Therefore, the present invention is available for development of drugs, health supplements or the like.

Best Mode for Carrying out the Invention

[0007]

The present invention is explained below in details by the embodiments (representative examples), but not

specified by those contents.

The essence of the present invention is based on a finding that saliva, a noninvasive collectable specimen, is useful for inspecting a level of in vivo migration of nutrition contained in a health supplement or the like in ingestion of the health supplement or the like. In the present invention, conduct to measure and methods to measure are referred to analysis methods respectively, and values obtained by analysis of individual sample and a plurality of samples are referred to measured values. And to determine a level of in vivo migration by comparing to amount of control are referred to inspecting.

In addition, "nutrition contained in a health supplement or the like" in the present invention mean the fat-soluble vitamins and/or fat-soluble food factors. And, for the fat-soluble vitamins and/or fat-soluble food factors used in the present invention, followings are exemplified. The fat-soluble vitamins include particularly tocopherol of vitamin E and β-carotene of provitamin A. The fat-soluble food factors include CoQ10 which can be synthesized in living bodies and lycopene which is an antioxidant, is not required to ingest because it is not a vitamin, but preferably is aggressively ingested to maintain and promote health. The specimen is sampled saliva, in particular preferably parotid saliva. This parotid saliva is sampled under a more selected environment

to give a more correct value. Approximately 70% of saliva is secreted from the sublingual gland and the submandibular gland, and another approximately 20% is secreted from the parotid gland. The object of the present invention can be achieved by sampling this parotid saliva more selectively. For this sampling, a saliva collecting tool already marketed (e.g. Saliva-sampler (Saliva Diagnostic Systems Inc.), Orasure (Orasure Technologies, Inc., Translated National Publication of Patent Application No. Hei 5-506925), Salivet (Assist Co., Ltd.)) can be used, and the tool has preferably a shape that at least allows more selective contact with the site of parotid saliva. For example, the flattened sampling part is more preferable for collecting saliva. Parotid saliva is collected by inserting an absorber between the teeth and the inside of the cheek, for example. The amount of collected saliva depends on types of the absorber of the collecting tool, and it is indispensable for obtaining relative values that a specific absorber is used, and a certain amount of saliva is brought contact with a certain amount of the absorber which adsorbs fat-soluble vitamins and/or fat-soluble food factors. That means, it is necessary that the collector takes care to keep the ratio of the amounts of the absorber and the contacting saliva and regulates the amount of the used absorber so that a solvent may be added to recover the analysis sample as a liquid. Concretely, the absorber is

preferably used with irreversibly absorbed saliva, and includes an absorber using capillary phenomenon.

The collecting tool may be supplied with a function like a collecting indicator to keep a constant collection amount. This indicates, for example, that the amount of the collected saliva may be confirmed by any means such as capillary phenomenon. For the absorber, any material is usable as far as it absorbs water and is insoluble in alcohols like ethanol. An artificial polymer material such as polyester-fiber or urethane foam and a natural material such as absorbent cotton, paper or pulp, can be used, and a cotton material is most suitable. Additionally, the collecting tool may be supplied with a storage container part for preserving the absorber with absorbed saliva. Furthermore the storage container part may hold a saliva preservative solution to dip and preserve the absorber. The preservative solution may be any water-soluble organic solvent. Lower alcohols such as ethanol, methanol or isopropanol are exemplified. For an analysis laboratory to take the collecting tool with collected saliva from a subject by mail, the tool holds desirably ethanol in a concentration so as to be excluded from a combustible material according to the postal regulation or the like. Also, the collecting tool may be any possible posting form, but is desirably a form which can be sent in a standard-size. The parotid saliva specimen, which is little affected by

a physiological condition, is preferably collected between meals under a stable condition of saliva secretion, in particular desirably two hours or longer after meal, rather than immediately after meal under a condition of enhanced saliva secretion.

[0008]

Collected saliva remaining in the collecting tool is preserved in the storage container and sent to an analysis laboratory or the like for analysis. In order to analyze the fat-soluble vitamins and/or fat-soluble food factors as target substances, the fat-soluble vitamins and/or fat-soluble food factors are just extracted and separated from the collecting tool, and quantified. Specifically, the method comprises: (a) a process for solvent-processing the collected saliva, the absorber with absorbed saliva and/or the preservative solution with the preserved absorber to extract the fat-soluble vitamins and/or the fat-soluble food factors, (b) a process for separating the fat-soluble vitamins and/or the fat-soluble food factors from the extract by high-performance liquid chromatography or the like, and (c) a process for detecting and quantifying the separated fat-soluble vitamins and/or the fat-soluble food factors by a known analysis method suitable for microassay, for example, using a UV detector or an electrochemical detector (ECD).

The preferable solvent is a solvent which can remove

a protein and extract the target fat-soluble vitamins and/or fat-soluble food factors from the collected saliva, the absorber with the absorbed saliva and/or the preservative solution with the preserved absorber. The above-mentioned water-soluble solvent is more preferable, and a water-soluble solvent having a concentration suitable for extraction, specifically, ethanol having a final concentration of at least 75%, is most suitable. The other solvent includes methanol and isopropanol available for the ECD which is more sensitive than the UV detector.

In order to extract, concretely, an absorber with 1 ml of saliva absorbed is supplied with a volume three times or more (approximately 66% or more) of ethanol as a solvent to contact. isopropanol and methanol having a volume five times or more relative to saliva should be contacted. The compounds are promptly extracted from the absorber, and they are not adsorbed again even after a long time. Absorbent cotton is suitably used by approximately 250 mg relative to 1 ml of saliva. The extracted specimen is analyzed, unprocessed or preprocessed by centrifugation for removing an agglomerated protein to recover the supernatant.

[0009]

Numerical values (concentrations) in saliva indicate in vivo migration of the fat-soluble vitamins and/or the fat-soluble food factors contained in the ingested health

supplements, drugs or foods. For example, these values are compared with the values before ingestion and it is allowable to inspect in vivo a level of migration of the fat-soluble vitamins and/or the fat-soluble food factors, and thereby it is available to assess the health situation, absorbability, nutritional ability or the like, and also it provides a method of inspecting on sick condition in the subjects.

Furthermore, these analysis methods allow tracing in vivo migration of the ingested fat-soluble vitamins and/or fat-soluble food factors and are available as methods for drug discovery or screening of novel health supplements.

Examples

[0010]

Hereinafter, the present invention will be described with examples. In the following examples, as representative examples of nutritional components contained in health supplements or the like, CoQ10 and tocopherol are used to confirm the effect, but the present invention is available for fat-soluble vitamins and/or fat-soluble food factors, and scope of the invention is not limited to these examples. In examples, CoQ10, tocopherol, lycopene and β-carotene were analyzed as follows.

[0011]

(Analytical condition)

HPLC was conducted in reference to a known method of an analysis method of CoQ10 in plasma (Analytical Biochemistry Vol. 250, p66-73, 1997), and improved so as to be suitable for saliva analysis.

[0012]

Pretreatment and Loading to an Autosampler

In pretreatment of the specimens, 20 μl of plasma and 180 μl of ethanol (Wako Pure Chemical Industries, Ltd.: HPLC grade) were added to an autosampler vial (sampler vial PP 250 μl: Shiseido Co., Ltd.) to have a final ethanol concentration of 90% (V/V), which was centrifuged under cooling by 1000 G for 5 minutes to precipitate proteins. In the case of directly collected saliva, 150 μl of ethanol was added to 50 μl of the specimen to have a final ethanol concentration of 75 %(V/V), which was centrifuged under cooling by 1000 G for 5 minutes to precipitate proteins. In the case of parotid saliva collected using the Saliva-sampler (produced by Saliva Diagnostic Systems Inc.), as the sampler adsorbs 1 ml of saliva as a fluid volume, 3 ml of ethanol (HPLC grade) was added to the sampler, and mixed by flipping upside down for 15 minutes, subsequently 200 μl was fractionated to each autosampler vial (sampler vial PP 250 μl: Shiseido Co., Ltd.), and centrifuged under cooling by 1000 G for 5 minutes to precipitate proteins. The centrifuged autosampler vial was loaded into the

autosampler and subjected to high-speed chromatography (HPLC).

[0013]

(Column in Use)

The following three columns were prepared for HPLC.

1. Concentrator column: CAPCELL PAKC18 AQ 5 μm ϕ 2.0 × 35 mm

2. Reduction column: SHISEIDO CQ ID 2.0 mm × 20 mm

3. Separation column: CAPCELL PAKC18 AQ 3 μm ϕ 2.0 × 150 mm

The concentrator column is used for concentrating the analysis target components in the sample and for removing hydrophilic foreign substances. The reduction column is used for reducing ubidecarenone (CoQ10) to ubidecarenol. A reduction is useful especially for simultaneous analysis of CoQ10 together with fat-soluble vitamins and/or the fat-soluble food factors with OH groups such as tocopherol, lycopene or β-carotene by using Electric Chemical Detector(ECD). The Separation column is used for purifying, refining, and detecting each component as a single component.

[0014]

(Mobile phase)

The followings were prepared as a developing solvent for the column.

Mobile phase 1: 6.122 g of an anhydrous sodium perchlorate

is mixed to dissolve in 950 ml of methanol and 50 ml of distilled water, and then deaerated under vacuum with ultrasound.

Mobile phase 2: 6.122 g of an anhydrous sodium perchlorate is mixed to dissolve in 950 ml of methanol and 50 ml of isopropanol, and then deaerated under vacuum with ultrasound.

Mobile phase 1 is used for sending the injected sample to the concentrator column to keep the analysis target component in the column, removing hydrophilic foreign components such as proteins, polypeptides, amino acids, organic acids, saccharides and salts which could not completely removed from the analysis sample by a pretreatment, and effectively rinsing away hydrophobic non-analysis target components such as cholesterols, fatty acids, phospholipids and neutral fats contained in the biological sample in a large quantity. Mobile phase 2 is used for passing the liquid through the separation column from the time of injection to completion of removal process of foreign components in the concentrator column, and on completing the removal process, then column-switching to flow reversely to the concentrator column, thereby to elute promptly the analysis target component and simultaneously sending the analysis target component to the separation column to purify, refine, and fractionate as a single component.

[0015]

In preparation of mobile phase 1, it should be noted that the mixture ratio of distilled water to methanol is increased to cause difficulty to remove the hydrophobic foreign components, and that the mixture ratio is decreased to cause difficulty to remove the hydrophilic foreign components. In mobile phase 2, even if the mixture ratio of isopropanol to methanol is increased from 95:5 to 90:10 or 80:20, it does not bring a significant influence on elution from the concentrator column and separation of the components in the separation column. The anhydrous sodium perchlorate is added by 6.122 g to prepare a concentration of 50 mM, and it may be by 6 g or 7 g.

[0016]

(Separation method)

The relation of interconnection among the separation system-constituting device, the mobile phase and the columns during 2 minutes from a start of sample-injection is shown in Fig. 1.

For solution sending in the mobile phases 1 and 2, a total of two pumps, pump 1 and pump 2 respectively (trade name: Pump 3001, Shiseido Co., Ltd.), are used. The flow rates are 200 μl/min. for the mobile phase 1 and 400 μl/min. for the mobile phase 2 from the start of analysis to the end. During analysis, a mobile phase, which is previously passed through a three pathway-type vacuum deaeration

device (trade name: SSC3215, Senshu Kagaku Co. Ltd.), is passed to cause no bubbles in the mobile phases 1 and 2. Furthermore, the reduction column and the separation column are tandemly connected and maintained at a constant temperature of 40°C in a column oven (trade name: Type 3004, Shiseido Co., Ltd.). For hexagonal bulbs 1 and 2, a rinse bulb (trade name: Type 3034, Shiseido Co., Ltd.), and a high-voltage switching hexagonal bulb (trade name: Type 3011, Shiseido Co., Ltd.) are used respectively. For the autosampler, HTS autosampler (Shiseido Co., Ltd.) is used, and for control management of programming the whole system and process of numerical values, EZChrom (Shiseido Co., Ltd.) is used.

The sample and the reference standard are injected using 20μl autosampler (trade name: HTS autosampler, Shiseido Co., Ltd.). Switching of passage is programmed using a system controller (trade name: EZChrom, Shiseido Co., Ltd.) which can automatically separate and analyze.

As the summary, for 2 minutes from a start of sample-injection, the program are arranged to be bulb position A, solution sending in the mobile phases 1 are executed by a speed of 200μl/minute, and thereby concentrated-purification of assay-subject have been achieved. During the executing, the mobile phases drawn away from the concentrator column are discharged to drain together with contaminants contained in sample, and a

piping of the assay system are arranged for them to be excluded to be introduced to the reduction column and the concentrator column. For 2 minutes from a start of sample-injection, a solution sending of the mobile phases 2 is executed by a speed of 400μl/minute to the reduction column and the concentrator column.

The relation of interconnection among the separation system-constituting device, the mobile phase and the columns during 2 ~15 minutes from the time of injection is shown in Fig. 2.

As the summary, 2 minutes later from a start of the assaying, the program are arranged to be bulb position B, solution sending of the mobile phases 2 are executed by a speed of 400μl/minute to the concentrator column from the opposite side in case of the sample-injection , and thereby assay-subject have been dissolved out. The dissolved assay subject is charged on reduction column and are reduced CoQ10 to Ubiquinol, and further the reduced substance are introduced to the concentrator column and the assay-subject components are separated. The separated each component is assayed each amount. And, each dissolving out - time of Ubiquinol and CoQ10 is about 14 minutes and about 26 minutes. In the present invention, the assay time have been reduced by introducing of a method which is assayed after changing to Ubiquinol. And also, during 2~15 minutes after charging of sample, a piping of the assay system are arranged for

them to be excluded to be introduced to any columns, and solution sending in the mobile phases 1 are executed by a speed of 200μl/minute from drain via Autosampler. During 15~17 minutes after charging of sample, a piping of the assay system are arranged for program to be changed to bulb position A. And it brings re-equilibrium for continuous assaying several samples. And it is showed by the relation of interconnection among the separation system-constituting device, the mobile phase and the columns in fig. 1.

For solution sending in the mobile phases 1 and 2, a total of two pumps, pump 1 and pump 2 respectively (trade name: Pump 3001, Shiseido Co., Ltd.), are used. The flow rates are 200 μl/min. for the mobile phase 1 and 400 μl/min. for the mobile phase 2 from the start of analysis to the end. During analysis, a mobile phase, which is previously passed through a three pathway-type vacuum deaeration device (trade name: SSC3215, Senshu Kagaku Co. Ltd.), is passed to cause no bubbles in the mobile phases 1 and 2. Furthermore, the reduction column and the separation column are tandemly connected and maintained at a constant temperature of 40°C in a column oven (trade name: Type 3004, Shiseido Co., Ltd.). For hexagonal bulbs 1 and 2, a rinse bulb (trade name: Type 3034, Shiseido Co., Ltd.), and a high-voltage switching hexagonal bulb (trade name: Type 3011, Shiseido Co., Ltd.) are used respectively. For the

autosampler, HTS autosampler (Shiseido Co., Ltd.) is used, and for control management of programming the whole system and process of numerical values, EZChrom (Shiseido Co., Ltd.) is used.

[0017]

(Detection method)

Ubidecarenol, tocopherol, lycopene and β-carotene can be quantitatively analyzed at 600 mV. CoQ10 with a carbonyl group, and tocopherol, lycopene and β-carotene with OH groups coexist in saliva. Therefore, CoQ10 is reduced the carbonyl group to the OH group using the above-mentioned commercial online reduction column, and then detected in an oxidation mode using the amperometry-type electrochemical detector 3005 (Shiseido Co., Ltd.) or the pulse-type electrochemical detector 3016 (Shiseido Co., Ltd.) under the HPLC condition shown in Figs 1 and 2, allowing the bulk detection as shown in Figs. 3 and 4.

The ECD was used after Sensitivity was set to 0.1 and Time Constant to a standard (Std.), and a maintained baseline level of approximately 1 nA was confirmed for every analysis. UV detectors are useful for analysis of a tocopherol, cholesterol, neutral fat and fatty acid being rich in sample. For the detection wavelength to set, a known wavelength is sufficient.

[0018]

(Calibration curve, sensitivity and injection volume of the sample)

When the amperometry-type electrochemical detector 3005 (Shiseido Co., Ltd.) or the pulse-type electrochemical detector 3016 (Shiseido Co., Ltd.) was used, 20 μl of reference standard mixtures of CoQ10, tocopherol, ubidecarenol, lycopene and β-carotene which were prepared to have their respective concentrations of 1.56 to 200 ng/ml, were injected to make a calibration curve capable of linear regression. When the HTS autosampler (Shiseido Co., Ltd.) is used as an autosampler, the injection volume of a sample is normally 20 μl, but a concentrated sample is injected by 2 μl, and a diluted sample is injected by 50 μl to separate and quantify. The concentration rate of a pretreated sample can be raised and the injection volume of a sample can be increased to change sensitivity to a desirable level.

[0019]

Example 1

Chromatograms of the samples of the parotid saliva specimens A, B and C are shown in Fig. 3, and a magnified view of Fig. 3 is shown in Fig. 4 so that the peaks of lycopene, β-carotene and CoQ10 are easily visible. Parotid saliva was collected from a volunteer who was taking two CoQ Livlon tablet (trade name, Nissin pharma Inc.)in twice/day, a commercial CoQ10 supplement, at 7:00 every morning and

evening for a month, and then 1 ml of the saliva was supplied with 3ml of ethanol for removing proteins to prepare a supernatant, 50 μl of which was injected to obtain the chromatogram of the sample of the parotid saliva specimen A. Contents in one tablet was assayed by injecting 20μl of supernatant removed proteins by adding of isopropanol 90ml after solving gelatin capsule by supersonic-wave treatment of 30minutes at 40 degree in centigrade against one tablet/10ml of distilled water. 24.1 mg/one tablet as for CoQ10, and 6.9 mg/tablet as for α-tocopherol were contained. And lycopene and beta-carotene were not detected.

The intakes from the supplement per day are 48.2 mg as for CoQ10, and 13.8 mg as for α-tocopherol. On the other hand, 1 ml of parotid saliva from a volunteer who took no commercial supplement was supplied with 3 ml of ethanol for removing proteins to prepare a supernatant, 50 μl of which was injected to obtain the chromatogram of the sample of the parotid saliva specimen B. Furthermore, parotid saliva was collected from a volunteer who was taking one commercial tomato juice can (180 ml) everyday and took a specified bland (trade name: Fully-Ripened Tomato, salt-free, Delmont, LTD.) for a week, and then 1ml of the parotid saliva was supplied with 3 ml of ethanol for removing proteins to prepare a supernatant, 50 μl of which was injected to obtain the chromatogram of the sample of

the parotid saliva C. Contents in one can was assayed by injecting 20μl of supernatant removed proteins by adding of isopropanol 9ml against 1ml of juice. The label showed that one can contained lycopene 19.7 mg and β-carotene 2.7 mg.

Comparing the peaks of tocopherol and CoQ10 in the chromatogram of the samples of parotid saliva A and B in Fig. 3, the A in a volunteer who was taking supplements shows a higher peak than the B. Additionally, comparing the chromatograms of the samples of parotid saliva B and C in Fig. 4, the peaks of lycopene and β-carotene in the person who drinks tomato juice regularly are obviously higher. Consequently, a volunteer with a higher intake showed a higher concentration in parotid saliva, and hence analysis of fat-soluble vitamins and/or fat-soluble food factors in saliva is useful for assessment of degree of in vivo migration of fat-soluble vitamins and/or fat-soluble food factors contained in ingested health supplements, medical drug or food.

[0020]

Example 2

11 volunteers were made to ingest orally two tablets (CoQ10 content: 100 mg in two tablets) of Q10AA Multi (trade name, Shiseido Co., Ltd., hereinafter may be referred to "Supplement S"), a commercial CoQ10 supplement, at 9:00 every morning continuously for a week. Then saliva and

heparinized plasma as samples were collected at PM 4:00, before and one week after ingestion of CoQ10. The volunteer had the mouth rinsed with water, and kept quiet on a chair for 5 minutes, while the volunteer accumulated saliva in the oral cavity and discharge to initialize the oral cavity, followed by collecting saliva. Approximately 1 ml of saliva accumulated in the oral cavity was collected as directly collected saliva. In order to collect saliva from the parotid gland, an absorbent cotton of 2 g was put beneath the tongue to prevent contamination with saliva from sublingual gland and submandibular gland, while the Saliva-sampler comprising a cotton saliva absorber and an indicator which informed termination of collection by color change was inserted between the teeth and the cheek near the open of the parotid gland. The volunteer sat quiet on a chair, during which saliva was collected. For the 11 volunteers, the mean amount of saliva collected using the Saliva-sampler was 1 ml, and the fluctuation range was 0.9-1.1 ml with good reproducibility. In analysis of CoQ10 in saliva collected from the parotid gland, the Saliva-sampler with absorbed saliva was transferred to the peculiar tube, to which 4 ml of ethanol was added to have a final concentration of 75%. The resultant solution was mixed by flipping upside down for 10 minutes to extract from the absorber and coagulate proteins, and then centrifuged by 1000G at 4°C to remove fragments of the absorber and the

unnecessary proteins to get a supernatant, 20 μl of which was subjected HPLC. Meanwhile, 2 g of absorbent cotton put beneath the tongue is not necessary for collecting of parotid saliva, and contamination of saliva from the sublingual gland and the submandibular gland can be avoided by swallowing. In pretreatment of directly collected saliva, 250 μl of the specimen was supplied with 750 μl of sample ethanol to mix, and centrifuged at 4°C by 1000 G for 5 minutes for removing proteins to get a supernatant, 20 μl of which was subjected to HPLC. In pretreatment of plasma, 20 μl of the plasma was supplied with 180 μl of ethanol to mix, and then centrifuged at 4°C by 1000 G for removing unnecessary proteins to get a supernatant, 20 μl of which was subjected to HPLC.

[0021]

Example 3 (Experiment of the correlation between the blood level and the content in saliva)

A good correlation was confirmed between the concentrations of CoQ10 in parotid saliva and blood of the 11 specimens after ingestion of CoQ10 and before ingestion of CoQ10. CoQ10 was orally ingested by 100mg/date every 9 AM during one week. Assay of CoQ10 was executed before ingestion and after one week of ingestion. As shown in Fig.5 and 6, there was a recognizable correlation between the CoQ10 levels in directly collected parotid saliva and plasma. Thereby, it was confirmed that analysis of the

values in parotid saliva, could correctly assess the amount of in vivo migration of nutritional supplements or the like.

[0022]

Example 4 (Assay in parotid saliva and saliva under tongue)

A correlation was studied between the concentrations of CoQ10 in parotid saliva or the concentrations of CoQ10 in saliva under tongue and blood of the 11 specimens after ingestion of CoQ10 and before ingestion of CoQ10. CoQ10 was orally ingested by 100mg/date during one week. Assay of CoQ10 was executed before ingestion and after one week of ingestion. As shown in Fig.5 and 6, there was a recognizable correlation between the CoQ10 levels in directly collected parotid saliva and plasma. However, as shown in Fig.7 and 8, there was not a recognizable correlation between the CoQ10 levels in directly collected saliva under tongue and plasma. Thereby, it was confirmed that analysis of the values in parotid saliva, could correctly assess the amount of in vivo migration of nutritional supplements or the like.

[0023]

Example 5 (The absorbent materials for parotid saliva and necessity of extraction)

In the method of preparing the specimens in example 2, the absorbent materials for saliva were weighed. 50 mg of an absorbent was soaked in 600 μl of saliva, 20 μl of

saliva was fractionated to analyze the CoQ10 concentration in saliva transuaded from the absorbent, to which 180 μl of isopropanol was added, mixed, and centrifuged at 4°C by 1000 G for 5 minutes to remove proteins, and 20 μl of the supernatant was subjected to HPLC. The results of analysis for CoQ10 are shown in Table 1 as "CoQ10 concentrations in saliva which was obtained by steps that 50 mg of the absorbent was soaked in 600 μl of saliva and the saliva was preserved in the interspace of the absorbent". It took about 3 minutes to secrete 1 ml of parotid saliva for the volunteers who spent little time, and 10 minutes or longer for the volunteers who spent lots of time, therefore it is practically difficult to collect without the absorbent. In this case, the fat-soluble food factors and the fat-soluble vitamins are adsorbed by any absorbent materials for saliva such as polyester, cotton and acetylcellulose. Although it is lowly absorptive, polyurethane fills water in saliva as bound water, so that condensation is caused, resulting in apparently high level (Refer to Table 1). Consequently, the adsorbed component should be extracted. For example, a cotton absorbent having contact with 2ml of saliva which contains 1 ml of water phase in the interspaces demonstrated that results of higher level can be obtained by extraction compared with the case of contact with 1 ml of saliva. That means, a certain amount of saliva should be absorbed to a certain

amount of absorbent in the oral cavity. Consequently, as for collection of parotid saliva, a material which the saliva fraction once absorbed into an absorbent such as the Saliva-sampler by capillary phenomenon does not leak from the absorbent, and a collecting tool which color change of an indicator or the like informs a correct collection amount, is used and it was executed by absorbing 1ml of parotid saliva.

[Table 1]

Treatment condition	CoQ10 (ng/ml)	Collection rate(%)
Saliva (control)	33.7	100
Cotton	3.4	10.2
Polyester	19.6	58.2
Polyurethane	59.8	178
Acetylcellulose	25	7.3

[0024]

Example 6 (Confirmation of extraction effects depending on deproteinization solvents)

In the method of preparing the specimens in examples 1 and 2, the solvents were weighed using ethanol (90, 75, 50%), isopropanol (90, 75, 50%) or methanol (90, 75, 50%). Saliva of 600 μl, and 600, 1800 and 5400 μl of the solvent

respectively were added to 50 mg of an absorbent to have a final concentrations of 50, 75 and 90% (V/V) respectively. The resultant solution was mixed, stirred, and centrifuged at 4°C by 1000 G to get a supernatant, 20 μl of which was used to analyze CoQ10 by HPLC. As a result, the values shown in the following Table 2 "The CoQ10 concentrations in the supernatant obtained by deproteinizing saliva in three solvents and at three concentration levels (ng/ml)" were obtained. Ethanol, which was not affected by adsorption to proteins even at a concentration of 75% and could efficiently extract, was confirmed to be most suitable.

[Table 2]

Concentration of the solvents used for deproteinization (V/V%)	Deproteinization solvents		
	isopropanol	ethanol	methanol
90	56	98	61
75	19	86	2
50	23	3	2

[0025]

Example 7 (Confirmation of extraction of analysis target component independent from the absorbent materials for

saliva)

In the method of preparing the specimens in example 2, the absorbent materials for saliva and the collection rate by extraction with 75% (V/V) ethanol were weighed. An absorbent of 50 mg was soaked in 600 μl of saliva, to which 180 μl of ethanol was added, mixed, and centrifuged at 4°C by 1000 G for 5 minutes to remove proteins, and 20 μl of the supernatant was subjected to HPLC. The results of analysis for CoQ10 are shown in Table 3 as "CoQ10 concentrations in saliva which was obtained by steps that 50 mg of the absorbent was soaked in 600 μl of saliva and the saliva was extracted with 1800 μl of ethanol (final concentration: 75%)". It was found that the CoQ10 concentration in saliva extracted at 75% (V/V) did not depend on the absorbent materials for saliva, and approximately 100% collection could be achieved at 75% (V/V). Thus, even if the fat-soluble components in saliva are adsorbed to the absorbent and contents of fat-soluble components in water phase preserved in the interspaces are reduced, the content in 1 ml of parotid saliva can be determined by analyzing the summation of the fat-soluble food factors and the fat-soluble vitamins contained in saliva preserved in the absorbent and interspaces. It has became that total amount is able to be recovered correctly by adding ethanol to amount of saliva absorbed by absorbent

[Table 3]

Treatment condition	CoQ10 (ng/ml)	Collection rate(%)
Saliva (control)	33.7	100
Cotton + ethanol	32.2	95.6
Polyester + ethanol	34.2	102
Polyurethane + ethanol	32.1	95.2
Acetylcellulose + ethanol	31.8	94.3

Brief Description of the Drawings

[0026]

[Fig. 1] It shows a relation of interconnection among a separation system-constituting device, a mobile phase and columns from injection of a sample to the time of concentration.

[Fig. 2] It shows a relation of interconnection among a separation system-constituting device, a mobile phase and columns from the time of elution through a concentrator column to the time of separation and refinement.

[Fig. 3] It shows chromatograms of the samples of a supplement-ingesting volunteer (saliva sample A), a

non-ingesting volunteer (saliva sample B) and a person who drinks tomato juice regularly (saliva sample C).

[Fig. 4] It shows a magnified view of peaks of lycopene and β-carotene by enlarging Fig. 3.

[Fig. 5] It shows correlation between the measured values of the CoQ10 in plasma and parotid saliva before ingestion of CoQ10.

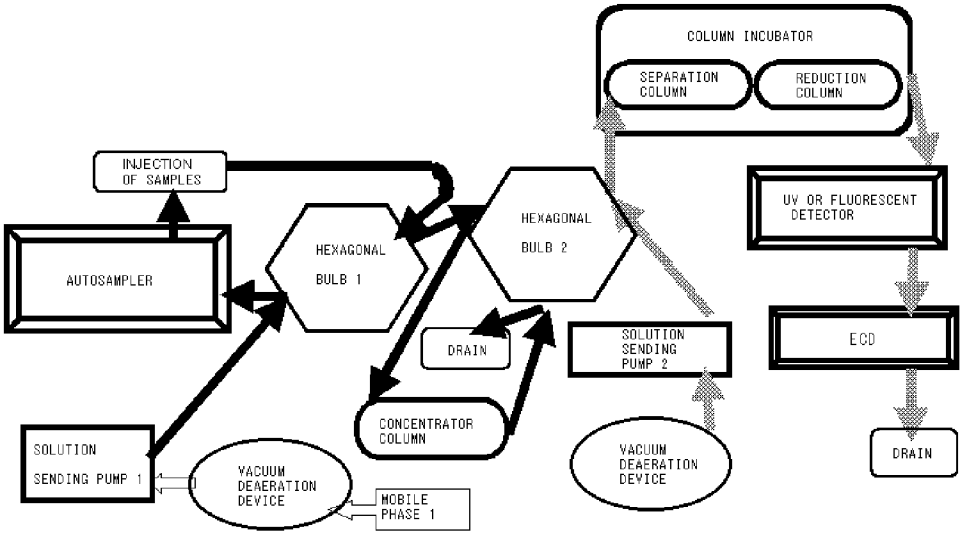
[Fig. 6] It shows correlation between the measured values of the CoQ10 in plasma and parotid saliva after ingestion of CoQ10.

[Fig. 7] It shows no-correlation between the measured values of the CoQ10 in plasma and directly collected saliva before ingestion of CoQ10.

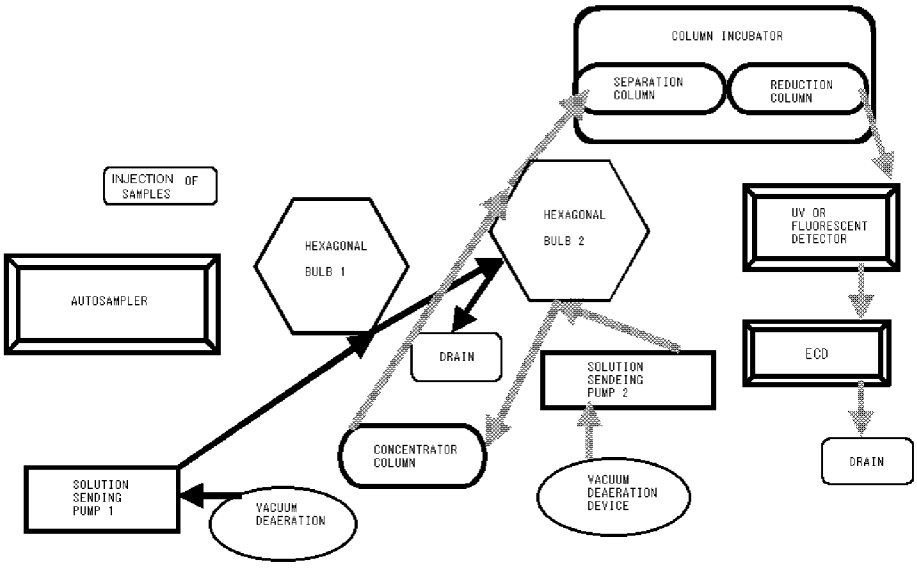
[Fig. 8] It shows no-correlation between the measured values of the CoQ10 in plasma and directly collected saliva after ingestion of CoQ10.

(Document name) Figures

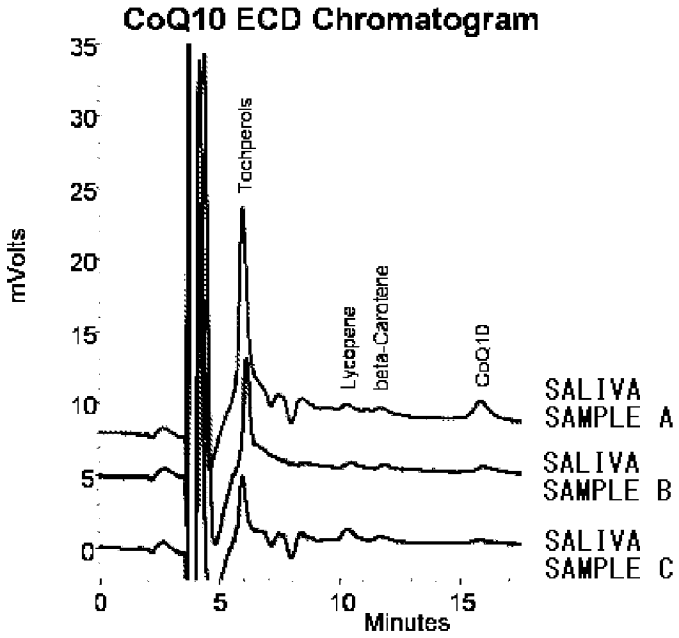
(Fig.1)



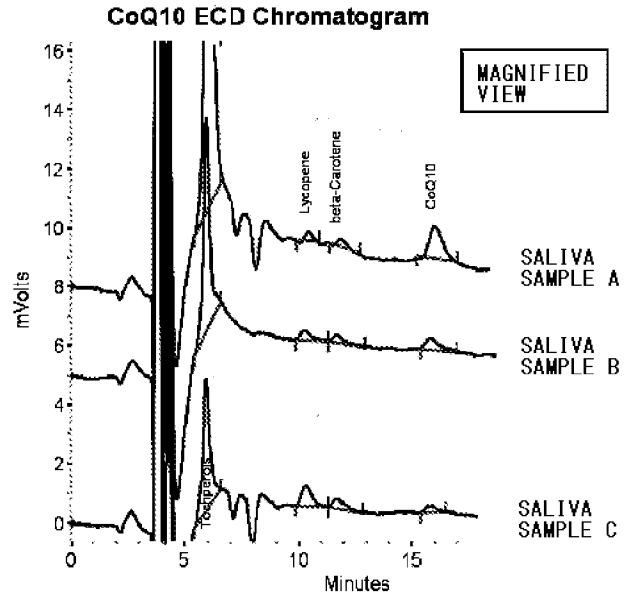
(Fig.2)



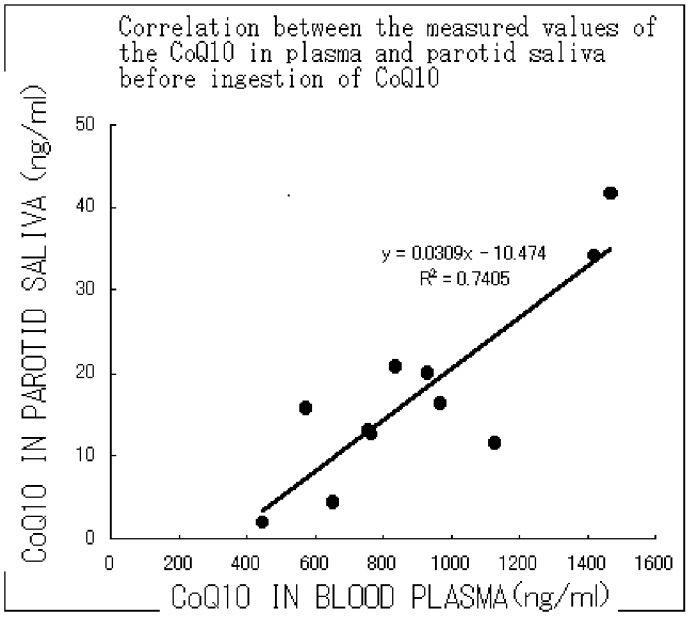
(Fig.3)


CoQ10 ECD Chromatogram
mVolts
35
30
25
20
15
10
5
0
Tochperols
Lycopene
beta-Carotene
CoQ10
SALIVA SAMPLE A
SALIVA SAMPLE B
SALIVA SAMPLE C
0
5
10
15
Minutes

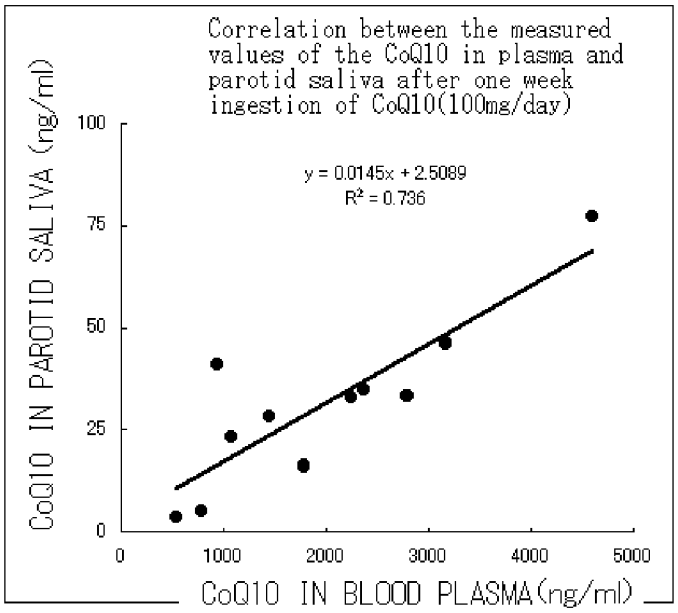
(Fig.4)


CoQ10 ECD Chromatogram
MAGNIFIED VIEW
mVolts
16
14
12
10
8
6
4
2
0
Lycopene
beta-Carotene
CoQ10
Tochperols
SALIVA SAMPLE A
SALIVA SAMPLE B
SALIVA SAMPLE C
0
5
10
15
Minutes

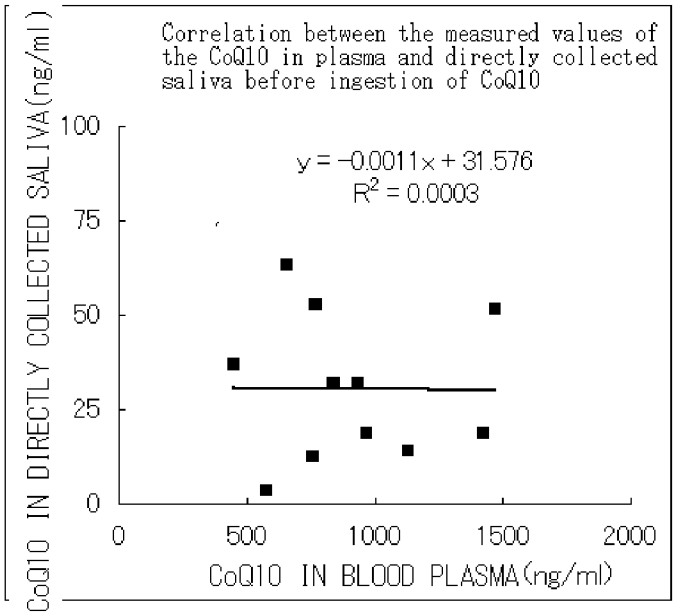
(Fig.5)


Correlation between the measured values of the CoQ10 in plasma and parotid saliva before ingestion of CoQ10
CoQ10 IN PAROTID SALIVA (ng/ml)
CoQ10 IN BLOOD PLASMA(ng/ml)
y = 0.0309x - 10.474
R² = 0.7405
0
10
20
30
40
50
0
200
400
600
800
1000
1200
1400
1600

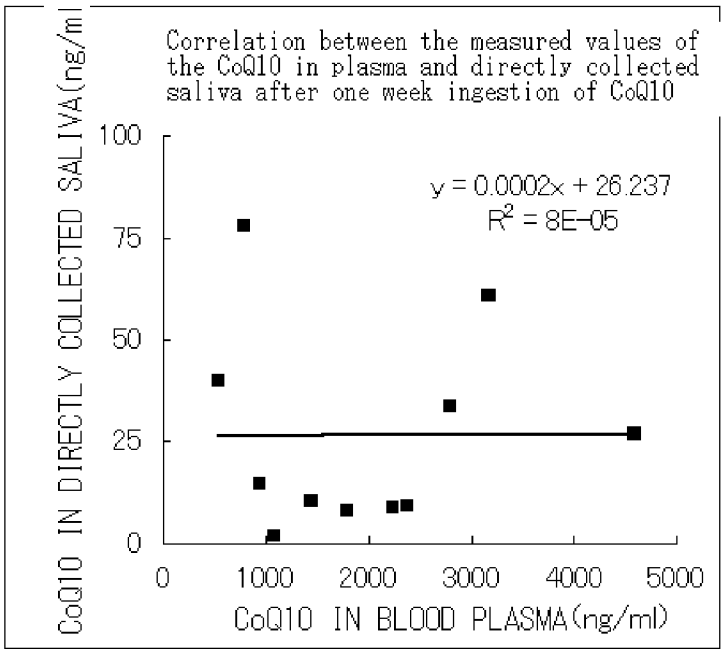
(Fig.6)


Correlation between the measured values of the CoQ10 in plasma and parotid saliva after one week ingestion of CoQ10(100mg/day)
CoQ10 IN PAROTID SALIVA (ng/ml)
CoQ10 IN BLOOD PLASMA(ng/ml)
y = 0.0145x + 2.5089
R² = 0.736
0
25
50
75
100
0
1000
2000
3000
4000
5000

(Fig.7)


Correlation between the measured values of the CoQ10 in plasma and directly collected saliva before ingestion of CoQ10
y = -0.0011x + 31.576
R2 = 0.0003
CoQ10 IN DIRECTLY COLLECTED SALIVA(ng/ml)
CoQ10 IN BLOOD PLASMA(ng/ml)
0
25
50
75
100
0
500
1000
1500
2000

(Fig.8)


Correlation between the measured values of the CoQ10 in plasma and directly collected saliva after one week ingestion of CoQ10
y = 0.0002x + 26.237
R2 = 8E-05
CoQ10 IN DIRECTLY COLLECTED SALIVA(ng/ml
CoQ10 IN BLOOD PLASMA(ng/ml)
0
25
50
75
100
0
1000
2000
3000
4000
5000

(Document name)ABSTRACT

(Abstract)

(Purpose) It is to present a simple way for assaying in vivo migration in the ingestion of a drug or a health supplement.

(Means of solving for the purpose) For a method for inspecting in vivo migration of fat soluble vitamins and/or fat soluble food factors in the ingestion of a drug or a health supplement, it is necessary to use saliva as a specimen, to contact a saliva collecting tool with a certain amount of saliva to absorb for collection, and to select a solvent for efficient extracting a measurement target component from the saliva collecting tool. The findings completed the present invention. Especially, it was found out that it is surely able for assay-subject in saliva to be followed even in a concentrated level of one/ten of blood. The findings completed the present invention. Accordingly, there are provided a method for inspecting in vivo migration of fat soluble vitamins and/or fat soluble food factors in the ingestion of a drug or a health supplement, comprising using saliva as a specimen to determine; the property of a saliva collecting tool; and a method for extracting from the saliva collecting tool.